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Genetic alteration of p53 resulting in loss-of-function is a common event in many human cancers. In contrast, the majority of human breast carcinomas express a wild-type p53 protein. p53 is a transcription factor which exerts its effects by regulating the expression of particular target genes. Alterations in the expression of factors that regulate p53-dependent transcription may contribute to breast carcinogenesis in which the p53 itself is wild-type. As an approach to identify such factors, three p53-dependent target genes have been identified in which additional sequence elements besides p53 binding sites are involved in the transcriptional response to p53.

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Resnick-Silverman, L. and Manfredi, J.J. (2000) Transcriptional regulation of a p53 response element in the p21 promoter is dependent on an upstream cis-acting element. 65th Symposium: Biological Responses to DNA Damage (Cold Spring Harbor, NY).

ANNUAL REPORT
Mechanisms of Breast Carcinogenesis Involving Wild-Type p53
CDA
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INTRODUCTION

The tumor suppressor protein p53 is a sequence-specific DNA-binding protein which is capable of regulating transcription both by activating and repressing mechanisms. These activities appear to be central to its role in a cellular checkpoint at the G1 to S phase transition in response to DNA damage caused either by ionizing or ultraviolet radiation or by various chemical means. Induction of DNA damage causes an increase in p53 levels resulting in activation of transcription of target genes including p21, an inhibitor of cyclin-dependent kinases, which results in either growth arrest or apoptosis. Consistent with its role as a tumor suppressor, a high frequency of mutation of the p53 gene has been reported in a variety of human cancers, particularly those of the colon, lung, and bladder. In such cases there is also loss of expression of the remaining allele of p53 such that only a mutant p53 is expressed. Similarly, breast cancers show a high frequency of loss of expression of one allele of p53 but the remaining allele is mutated in only 20-30% of the cases. These data suggested that either p53 is not directly relevant to the mechanism of oncogenesis in breast cancer or breast cancer cells may have alternate mechanisms for inactivation of p53 besides genetic alteration. The research funded by this award is centered on testing the hypothesis that there are novel mechanisms of carcinogenesis involving functional inactivation of this wild-type p53 that do not involve direct genetic alteration in this kind of tumor. As p53 is a transcription factor which exerts its effects by regulating the expression of particular target genes, alterations in the expression of factors that regulate p53-dependent transcription may contribute to breast carcinogenesis in which the p53 itself is wild-type. As a first step in identifying such factors, three p53-dependent target genes have been identified in which additional sequence elements besides p53 binding sites are involved in the transcriptional response to p53.

BODY

Results and Discussion

The two binding sites for p53 in the human p21 promoter are representative of two distinct classes of genomic p53 response elements.

Two response elements for the tumor suppressor p53 have been identified in the promoter of the gene encoding the cyclin-dependent kinase inhibitor p21. Binding of a monoclonal antibody directed against the carboxyl terminus of p53 selectively enhanced binding by p53 to the upstream (5′ site) without affecting the affinity of p53 for the downstream (3′ site). Mutational analysis showed that a single base change can cause one site to behave similarly to the other site. These sites in the *p21* promoter represent two distinct classes of p53 response elements found in a variety of genes. The mAb 421-enhanced class was shown to include elements from the *gadd45*, *mdm2*, and *cyclin G* genes as well as the box A element from the *IGF-BP3* gene whereas the mAb 421-inhibited class included *bax*, the 3′ element from the *p21* promoter, a novel element from the *cdc25C* gene, and the box B element from the *IGF-BP3* gene. These results

demonstrate the existence of two classes of p53 binding sites in the human genome and that the binding of p53 to these two classes of sites can be differentially regulated by binding of mAb 421. This is a novel example of the regulation of binding site selection by a transcription factor and suggests a possible mechanism for selectivity in target gene activation by the p53 protein.

To further examine the role of the C-terminal region in the differential binding of p53 to these two elements, truncated forms of p53 were produced either by proteolytic digestion of the full-length protein or by recombinant expression of a defined deletion mutant. While full-length p53 bound comparably to both sites, fragments of p53 lacking the carboxyl terminus showed enhanced binding to the *p21* 5′ site without substantially affecting binding to the *p21* 3′ site. Such assays can be used to classify other p53 response elements confirming that each of the sites in the p21 promoter are indeed representative of a distinct subset of p53 binding sites. These results demonstrate that the C-terminal region of p53 selectively negatively regulates the binding of p53 to elements such as the *p21* 5′ site and, consistent with previous studies, suggest that modification of the C-terminus is required to achieve high affinity binding to these sites in cells. Although such C-terminal modification can explain how p53 transcriptional activates via an element like the *p21* 5′ site, a distinct mechanism is clearly necessary for p53-dependent activation via elements such as the *p21* 3′ site.

The 3′ site from the human p21 promoter requires another sequence element to mediate p53-dependent activation.

p21 promoter constructs with deletion of the 20 bp containing either the 5′ or 3′ p53 response element reduced p53-dependent activation in a luciferase reporter, although clearly deletion of the 3′ site has a greater effect. This results demonstrates that the 5′ and 3′ sites cooperate in activation of the *p21* promoter. A single copy of the 5′ site placed upstream of a minimal adenovirus E1b promoter conferred strong (1200-fold) p53-dependent activation on the reporter whereas a single copy of the 3′ site conferred only weak (5-fold) p53-dependent activation. Two copies of the 3′ site conferred 315-fold activation demonstrating that the 3′ site is, indeed, a *bona fide* p53 response element. However, electrophoretic mobility shift assays (EMSA) showed that the binding of p53 to the 3′ site was no more that 3-fold weaker than the binding of p53 to the 5′ site. These results argue that in the context of the *p21* promoter the 20 bp contained in the 5′ site is sufficient for transcriptional activation by p53. In contrast, it is likely that the 3′ site must synergize with other activator response elements to mediate p53-dependent activation. Deletion analysis of the *p21* promoter has demonstrated that this is indeed the case. Deletion of 740 bp containing the 5′ site reduces p53-dependent activation in four different cell lines. The resulting construct contains the 3′ site and 190 bp of upstream sequence. Deletion of this 190 bp further diminishes p53-dependent activation even though the resulting construct still retains the 3′ site. This results suggests that this 190bp contains an element which cooperates with the 3′ site to mediate robust p53-dependent activation. 715 bp of the *p21* promoter containing the 3′ site and 695 bp of upstream sequence was inserted into a luciferase reporter containing a minimal adenovirus E1b promoter. Such a construct was activated 50-fold by p53 as compared to a construct containing only the 3′ site which was activated 5-fold. Deletion analysis demonstrated that as little as 20 bp of upstream sequence enhanced p53-dependent activation via the 3′ site. A construct containing only the 50 bp upstream of the 3′ site but lacking the 3′ site itself was not activated by p53 demonstrating that this upstream sequence does not contain an additional p53 response element. Hence, a sequence element located within the immediately adjacent 20 bp cooperates with the 3′ site to activate transcription in a p53-dependent manner.

There is a third p53 responsive element in the human p21 gene located in the first intron.

Sequencing of the region of human chromosome 6 which contains the human *p21* gene was recently completed (Genbank Accession Number Z85996). Comparison of this sequence with that of the cDNA for human *p21* has allowed the determination of the exon-intron structure of the human *p21* gene. The sequence of the entire gene was screened for potential p53 binding sites. One such site was identified in the first intron located at +3914 to +3933. An oligonucleotide containing this sequence was radiolabeled and shown to bind to purified p53 in an EMSA. Binding was competed with an excess of the unlabeled intronic site as well as the 5′ site, but was competed poorly by an oligonucleotide containing a mutated 5′ site. This demonstrates that p53 binds to this intronic site in a sequence-specific manner. Incubation with monoclonal antibodies resulted in the appropriate supershifted complexes and demonstrated that the binding of p53 to the intronic site was enhanced in the presence of mAb 421. This 20 bp intronic site was placed upstream of the minimal E1b promoter and shown to confer p53-dependent activation in both a p53-negative cell line upon co-transfection of wild-type p53 or in a wild-type p53-expressing cell line after DNA damage treatment. Thus, a third p53 response element exists in the first intron of the human *p21* gene.

The p53 response element in the human bax promoter consists of overlapping p53 binding sites.

Previous studies had identified a 36 bp element derived from the *bax* promoter that conferred p53-dependent activation on a reporter construct and that p53 bound in a sequence-specific manner to this element. This element contains three overlapping sequences each of which could potentially bind to p53. Oligonucleotides containing each of these were synthesized and shown to bind to p53 in a sequence-specific manner although the binding of p53 to these sites was relatively weak. Only one of the sequences conferred p53-dependent activation on a reporter, and this activation was less than that seen with the full-length 36 bp element. Further mutational analysis demonstrated that the minimal element which binds to p53 *in vitro* and confers p53-dependent activation on a minimal promoter as well as the full-length 36 bp element is contained within -113 to -83 which contains only two of the three binding sites. These results indicate that activation of the *bax* promoter by p53 is mediated by cooperative interaction of two overlapping, low affinity, p53 binding sites. This is in marked contrast to other p53 target genes, particularly *p21,* in which single, high affinity p53 binding sites mediate the activation. Electrophoretic mobility shift assays showed that the full-length 36 bp element from the *bax* promoter as well as each of the three binding sites fall into the class of p53 binding sites that is inhibited by mAb 421. Thus, p53-dependent activation of the *bax* promoter is distinguished in two ways: by the synergy of overlapping low affinity binding sites and by its differential response to mAb 421.

A high-affinity Sp1 binding site is located within the p53-response element of the human bax promoter and contributes to p53-dependent activation.

Deletion of the -113/-83 sequence in a luciferase reporter containing the human *bax* promoter completely abolishes p53-dependent transcriptional activation. As noted, this 31 bp consists of three half-sites (RRRCWWGYYY) for p53 binding in a row. Deletion of the first or last half-site reduces p53-dependent activation demonstrating that all three half-sites are necessary for this response in the context of the *bax* promoter itself. Although the second and third half-sites can mediate some p53-dependent activity alone when placed upstream of the minimal E1b promoter, maximal activation

is only seen when all three half-sites are present. The first and second half-sites alone only confer robust p53-dependent activation when multimerized. Nevertheless, this result confirms that p53 bound to just the first two half-sites is capable of transcriptionally activating such a reporter. This latter suggests the possibility that the third half-site, although capable of interacting with p53, may serve as a binding site for another cellular factor that cooperates with p53 bound to the first two half-sites to mediate efficient transcriptional activation. Indeed, several such cellular factors have been identified. One of these is clearly the transcription factor Sp1. In EMSA, a DNA/protein complex is efficiently supershifted by an anti-Sp1 antibody and effectively competed by an unlabeled oligonucleotide containing the Sp1 consensus sequence for binding. The binding site for Sp1 was localized by further EMSA to the third-half site. To determine the relevance of this Sp1 binding site in p53-dependent transcriptional activation, 2 bp substitution mutants were constructed. One such mutant was shown to abolish an interaction of p53 with the third-half-site while retaining the ability to bind to Sp1 *in vitro*. A luciferase reporter containing these two substitution mutations was shown to confer 5-fold higher p53-dependent transcriptional activation on a minimal promoter than the unmutated element. Since this element can only bind p53 through the first and second half-sites, this demonstrates that the third-half site contributes to transcriptional activation by a mechanism that does not involve the direct interaction of p53. The most likely mechanism involves an interaction of some other cellular factor, possibly Sp1. The higher activation seen with the mutant suggests the possibility that p53 binding to the third-half site may interfere with this factor and hence an inability of p53 to interact with the third half site allows for more robust activation through the mutated element. Examination of the sequence of the third half-site shows that it resembles what is referred to as a "GC box". A large variety of known transcription factors interact with GC boxes, the most prominent of which is Sp1.

Cdc25C is a target for sequence-specific repression by the tumor suppressor protein p53.

The dual specificity phosphatase Cdc25C mediates cell cycle progression into mitosis and has been implicated as a key player in the G2 checkpoint in response to DNA damage. A binding site for p53 in the human *cdc25C* promoter is sufficient to confer p53-dependent activation. However, induction of p53 down-regulates expression of endogenous *cdc25C* RNA and protein. The element responsible for this p53-dependent repression is shown to consist of the p53 binding site and an overlapping binding site for Sp1. A 2 bp mutation outside the p53 binding site inhibits both the binding of Sp1 *in vitro* and p53-dependent repression in cells. These results are consistent with p53 repressing *cdc25C* expression by blocking the binding to the promoter of another factor, most likely Sp1. Repression of *cdc25C* by p53 is proposed to be an additional mechanism for p53-dependent arrest in response to DNA damage. Further this is the first demonstration of p53-dependent down-regulation of a physiologically relevant target that requires sequence-specific DNA binding by p53.

p53-dependent regulation of a subset of target genes is defective in four tumor cell lines.

Luciferase reporter plasmids containing either the *p21*, *bax*, or *PIG3* promoters were transfected into five p53-negative tumor cell lines. A published study from our laboratory demonstrated that in osteosarcoma Saos-2 cells, wild-type p53 effectively activated transcription of reporter constructs containing either the *p21* or *bax* promoters whereas wild-type p53 expressed in the breast carcinoma MDA-MB-453 cells, while still capable of activating transcription of a reporter containing the *p21* promoter, failed to

activate transcription through a construct containing the *bax* promoter. This previous result has been confirmed and defective *bax* activation has also been found in three other carcinoma cell lines, from lung, H1299, breast , MDA-MB-157, and ovary, SK-OV-3. In contrast, these cell lines have differing capacities for p53-dependent activation of the *PIG3* promoter. Interestingly, although p53-dependent repression via the *cdc25C* element is intact in four of the lines, the ovarian SK-OV-3 cells are defective for this repression. Levels of p53 expression in these transient assays was examined by immunoblotting of cell extracts. This demonstrated that these cell line differences can not be explained by level of p53 expression.

To determine whether the isolated p53 response element of the *bax* promoter was sufficient for this differential effect in Saos-2 and MDA-MB-453 cells, constructs containing isolated sites adjacent to the minimal E1b promoter were used. In Saos-2 cells, wild-type p53 effectively activated transcription of constructs containing either the 5' or the 3' sites from the *p21* promoter, as well as a construct containing the p53 element of the *bax* promoter. Similar to the observations with the promoter constructs, wild-type p53 expressed in the MDA-MB-453 cell line failed to activate transcription via a reporter containing the p53 response element of the *bax* promoter, while activating reporters containing either the 5' or the 3' element of the *p21* promoter. Expression of wild-type p53 in MDA-MB-453 cells also effectively activated transcription of reporters containing the p53 response elements of the *cyclin G* and *cdc25C* genes. Although p53-dependent activation via the *bax* element is defective in MDA-MB-453 cells, this does not rule out the possibility that other *bax* promoter elements are involved as well.

Adherence to Statement of Work

<u>Technical Objective #1</u>		
Task 1:	Months 1-5	In progress
Task 2:	Months 6-17	In progress
Task 3:	Months 18-27	In progress
<u>Technical Objective # 2</u>		
Task 4:	Months 1-5	Completed
Task 5:	Months 6-17	In progress
Task 6:	Months 18-23	Pending
Task 7:	Months 24-35	Pending
Task8:	Months 36-39	Pending
<u>Technical Objective #3</u>		
Tasks 9-11:	Months 13-39	Pending
<u>Technical Objective #4</u>		
Tasks 12:	Months 40-48	Pending

KEY RESEARCH ACCOMPLISHMENTS

- two binding sites for p53 in the human p21 promoter are representative of two distinct classes of genomic p53 response elements
- the 3′ site from the human p21 promoter requires another sequence element to mediate p53-dependent activation
- a third p53 responsive element in the human p21 gene is located in the first intron
- the p53 response element in the human bax promoter consists of overlapping p53 binding sites
- a high-affinity Sp1 binding site is located within the p53-response element of the human bax promoter and contributes to p53-dependent activation
- Cdc25C is a target for sequence-specific repression by the tumor suppressor protein p53
- p53-dependent regulation of a subset of target genes is defective in four tumor cell lines

REPORTABLE OUTCOMES

Manuscripts

1. Thornborrow, E.C. and Manfredi, J.J. (2001) The tumor suppressor protein p53 requires a cofactor to transcriptionally activate the human *bax* promoter. Submitted.

Abstracts

1. Thornborrow, E.C. and Manfredi, J.J. (2000) A high-affinity Sp1 binding site is located within the p53-response element of the human bax promoter. Tenth p53 Workshop (Monterey, CA).
2. St. Clair, S., Padi, A., Denburg, M., and Manfredi, J.J. (2000) Cdc25C is a target for sequence-specific repression by the tumor suppressor protein p53. Tenth p53 Workshop (Monterey, CA).
3. Manfredi, J.J., Thornborrow, E., St. Clair, S., Padi, A., Mattia, M., and Resnick-Silverman, L. (2000) *Cis*-acting promoter elements regulate gene expression mediated by the tumor suppressor p53. Era of Hope: Department of Defense Breast Cancer Program Meeting (Atlanta, GA).
4. Resnick-Silverman, L. and Manfredi, J.J. (2000) Transcriptional regulation of a p53 response element in the p21 promoter is dependent on an upstream cis-acting element. 65th Symposium: Biological Responses to DNA Damage (Cold Spring Harbor, NY).

Presentations

1. Regulation of target gene selectivity by the tumor suppressor p53
   Department of Pathology
   State University of New York at Stony Brook
   Stony Brook, NY
   January 13, 2000

2. Determinants of the cellular response to the tumor suppressor p53
   Department of Molecular Genetics, Biochemistry, and Microbiology
   University of Cincinnati Medical Center
   Cincinnati, OH
   October 24, 2000

Funding applied for based on work supported by this award

Title of Project:	Determinants of cellular responses to p53
Sponsoring agency:	National Institutes of Health/National Cancer Institute
Number:	1 R01 CA86001-01A1
Grant type:	Research
Status:	Principal Investigator
Percent Effort:	25%
Total Project Period:	04/01/01-03/31/06
Total direct costs:	$875,000

This application was reviewed by the Pathology B Scientific Review Group and received a priority score of 183 and a percentile of 23.9.

CONCLUSIONS

Studies identified three target genes for p53 in which an additional promoter element besides the p53 binding site itself is implicated in regulation of p53-dependent gene expression. This is consistent with the idea that p53 binding sites in the *p21, bax,* and *cdc25C* promoters each require *cis*-acting elements to confer efficient p53-dependent regulation and that cellular factors other than p53 are important for control of gene expression by p53. Should these *cis*-acting elements be demonstrated to be promoter-specific, this represents a mechanism for regulation of target gene selectivity by p53.

Because p53 is a tumor suppressor protein, mechanisms for regulating p53 represent mechanisms of carcinogenesis. By inhibiting the ability of the DNA damage signal to be translated into apoptosis, cells can sustain genetic lesions which can be propagated and result in oncogenic progression. Understanding how wild-type p53 can be functionally inactivated is therefore critical to our understanding of the molecular basis of a variety of human cancers. The research outlined in this proposal can serve as the basis for future clinical studies in both the prognosis and treatment of particular human tumors for two key reasons. First, such studies will allow determination of whether certain subtypes of breast cancer are linked with specific genetic alterations and if alterations in the expression or activity of factors that regulate the cellular response to p53 may be associated with a particular prognosis or a particular response

rate for a type of therapy. Second, the optimal therapeutic response to DNA damage caused by many chemotherapeutic agents used to treat breast cancer is apoptosis rather than cell cycle arrest. Elucidating the molecular mechanisms that are responsible for the ability of p53 to trigger apoptosis versus arrest may lead to more effective therapeutic intervention and a way to overcome the chemotherapeutic-resistant phenotype found in many breast tumors. Thus, elucidating mechanisms of breast carcinogenesis involving inactivation of wild-type p53 function, particularly resulting in a defective apoptotic response represents an important avenue in breast cancer research.

APPENDICES

The tumor suppressor protein p53 requires a cofactor to transcriptionally activate the human *bax* promoter

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Running title: p53-dependent transactivation of *bax*

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Summary

An important regulator of the proapoptotic *bax* is the tumor suppressor protein p53. Unlike the *p21* gene, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, it previously was reported that activation of the *bax* element by p53 requires additional sequences. Here, it is demonstrated that the minimal *bax* response element capable of mediating p53-dependent transcriptional activation consists of two p53 half-sites plus an adjacent six base pairs (5'-GGGCGT-3'). This GC-rich region constitutes a "GC-box" capable both of binding members of the Sp family of transcription factors, including Sp1 *in vitro* and of conferring Sp1-dependent transcriptional activation on a minimal promoter in cells. Mutations within this GC-box abrogated the ability of p53 to activate transcription without affecting the affinity of p53 for its binding site, demonstrating that these six bases are required for p53-dependent activation. In addition, a positive correlation was observed between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element *in vitro*. Mutations that inhibited Sp1 binding also blocked the ability of p53 to activate transcription through this element. Together, these results suggest a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human *bax* promoter.

Introduction

The bcl-2 family of proteins are key mediators of the apoptotic response. One member of this family is the proapoptotic Bax. Proceeding apoptosis, cytosolic Bax translocates to the mitochondria and homodimerizes. Homodimeric Bax then is thought to cause the release of cytochrome c (1-3) which subsequently functions as a coactivator of Apaf-1 in the cleavage of pro-caspase-9, initiating programmed cell death (4). Bax exists in equilibrium with two of its homologs, Bcl-2 and Bcl-xL. Unlike Bax, these two homologs exert antiapoptotic effects by heterodimerizing with Bax in the mitochondria, blocking its ability to release cytochrome c (5,6). Thus, an important determinant of the apoptotic response of a cell is the balance between the levels of Bax and Bcl-2/Bcl-xL. In this regard, regulation of the level of expression of Bax protein is key.

An important regulator of *bax* gene expression is the tumor suppressor protein p53 (7,8). The p53 protein has been implicated in several growth-related pathways, including apoptosis and cell-cycle arrest (9,10). The ability of p53 to function as a sequence-specific DNA binding protein appears to be central to its role as a tumor suppressor (11,12). At its amino-terminus, the protein contains a potent transcriptional activation domain (13) which is linked to a central core domain that mediates sequence-

specific DNA binding (14-16). Both of these domains have been shown to be important for p53-mediated growth suppression (17).

A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, and W is an adenine or thymine) separated by 0-13 bp, forming four repeats of the pentamer 5'-RRRCW-3' alternating between the top and bottom strands of the DNA duplex (18,19). Through sequences similar to this consensus, p53 has been shown to activate the transcription of many genes, including *bax*, *p21*, *mdm2*, *gadd45*, *IGF-BP3*, and *cyclin G* (8,20-26). When compared to alternate p53 targets, studies demonstrate that the *bax* gene is differentially regulated by wild-type p53 in a cell type-specific manner (7,27,28). In the mouse, p53-dependent regulation of *bax* expression following ionizing radiation is seen in the prostate, thymus, spleen, small intestine, and lung, as well as sympathetic, Purkinjie, and olfactory cortical neurons. In the kidney, heart, liver, and brain, however, no p53-dependent regulation of *bax* is observed (7,27). Further, the myeloid leukemia ML-1, Burkitt's lymphoma WMN and AG876, and lymphoblastoid NL2 and FWL cell lines induce *bax* following ionizing radiation, while the fibroblast AG1522 and WI38, colorectal carcinoma RKO, and osteosarcoma U2-OS cell lines fail to do so (28). In addition, several tumor-derived p53 mutants have been identified that are capable of

activating transcription through the promoter of the *p21* gene but not through the *bax* promoter (29-32). This correlates with an inability of these mutants to trigger apoptosis (29,31,32), suggesting that a failure in the ability of p53 to transactivate the *bax* gene may play an important role in tumor formation and progression. Supporting this, Yin *et al.* demonstrated that Bax is an obligatory downstream effector for the p53-mediated apoptosis that attenuates choroid plexus tumor growth in the TgT121 mouse model (33). Thus, a complete understanding of the transcriptional regulation of the *bax* promoter by p53 may yield important information relevant to our understanding of tumorigenesis.

Here is presented a detailed analysis of the p53 response element located in the promoter of the human *bax* gene. The minimal *bax* response element capable of mediating p53-dependent transcriptional activation is found to consist of two p53 half-sites plus an adjacent six base pairs (5'-GGGCGT-3') that demonstrate sequence-specific binding to the transcription factor Sp1. Mutational analysis of this "GC-box" shows it to be required for p53-dependent activation, and a positive correlation between the ability of p53 to activate transcription in cells and the ability of Sp1 to bind this response element *in vitro* is observed. These results are consistent with a model in which p53 requires the cooperation of Sp1 or a Sp1-like factor to mediate transcriptional activation of the human *bax* gene. This presents the intriguing possibility that regulation of this co-factor may

represent a novel basis for the cell-type specific control of the proapoptotic *bax* by wild-type p53.

Experimental Procedures

Cells

The osteosarcoma Saos-2 cell line was maintained in a humidified tissue culture incubator at 37°C with 5% CO_2. Cells were grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. *Drosophila* SL2 cells were cultured at 25°C in Schneider's Drosophila medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Oligonucleotides

For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the indicated sequences:

Bax-113/-77,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGCTA

G C G A A T T ; B a x - 1 1 3 / - 8 3 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAAT

T ; B a x - 1 1 3 / - 9 2 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; Bax-102/-83,

AATTCGGTACCAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; Bax-113/-83(sc-102/-93), AATTCGGTACCTCACAAGTTAGCTCACCTAAGGGGCGTGGGCGCTAGCGAATT; Bax(-113/-93)3, AATTCGGTACCTCACAAGTTAGAGACAAGCCTCACTGGTCACAAGTTAGAGACAAGCCTCACTGGTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; Bax-92/-83, AATTCGGTACCGGGCGTGGGCGCTAGCGAATT; p21-5', AATTCGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; Bax/p21-5' hybrid, AATTCGGTACCAGACAAGCCTCAACATGTTGGCTAGCGAATT; p21-5'/Bax hybrid, AATTCGGTACCGAACATGTCCGGGCGTGGGCGCTAGCGAATT; Sp1 Consensus, ATTCGATCGGGGCGGGGCGAGC; BaxGG-92/-91AA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTAAGCGTGGGCGCTAGCGAATT; BaxGG-85/-84AA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGAACGCTAGCGAATT; BaxG-92A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTAGGCGTGGGCGCTAGCGAATT; BaxG-91A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGAGCGTGGGCGCTAGCGAATT; BaxG-90A, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGACGTGGGCGCTAGCGAAT

T ; Bax C-89A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGAGTGGGCGCTAGCGAAT

T ; Bax G-88A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCATGGGCGCTAGCGAAT

T ; Bax T-87G ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGGGGGCGCTAGCGAAT

T ; Bax G-86T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTTGGCGCTAGCGAAT

T ; Bax G-85T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGTGCGCTAGCGAAT

T ; Bax G-84T ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGTCGCTAGCGAAT

T ; Bax C-83A ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGAGCTAGCGAAT

T ; Bax sc-92/-83 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTTTTATGTTTAGCTAGCGAATT

; Bax sc-86/-83 ,

AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTTTTAGCTAGCGAAT

T.

Plasmids

The following synthetic double-stranded oligonucleotides were digested with KpnI and NheI and cloned into pGL3-E1bTATA (34), which also had been double-digested with KpnI and NheI to produce pTATA vectors with corresponding names: Bax-113/-83, Bax-113/-93, Bax-102/-83, Bax-113/-83(sc-102/-93), Bax(-113/-93)3, Bax-92/-83, Bax-133/-77, p21-5', BaxGG-92/-91AA, BaxGG-85/-84AA, BaxG-92A, BaxG-91A, BaxG-90A, BaxC-89A, BaxG-88A, BaxT-87G, BaxG-86T, BaxG-85T, BaxG-84T, BaxC-83A, Bax sc-92/-83, Bax sc-86/-83. pBax-315/+51, pBax-127/+51, and pBax-76/+51 were generated by PCR amplification of the appropriate fragments from the original pBax luciferase reporter plasmid (8). Upstream primers were engineered with the NheI restriction site. Downstream primers contained the HindIII restriction site. Following PCR, products were digested with both NheI and HindIII and cloned into pGL3-E1bTATA which was also double digested with NheI and HindIII, removing the adenovirus *E1b* minimal promoter. To construct pBaxΔ-126/-77, PCR amplification of the original pBax was used to generate two fragments corresponding to -315 to -127 and to -76 to +51 from the start site of transcription. The -315 to -127 fragment was engineered to contain the NheI restriction site on the upstream side and the SacI restriction site on the downstream side. The -76 to +51 fragment was engineered to contain the SacI site upstream and the HindIII site downstream. Following PCR amplification each fragment was double-digested with the appropriate restriction

enzymes (NheI and SacI or SacI and HindIII). A three way ligation with the two PCR-generated fragments and pGL3-E1bTATA, double-digested with NheI and HindIII, then was performed, replacing the *bax* sequence from -126 to -77 with the SacI restriction site. To construct pBaxΔ-113/-104, PCR amplification of the original pBax was used to generate two fragments corresponding to -315 to -114 and to -103 to +51 from the start site of transcription. The -315 to -114 fragment was engineered to contain the NheI restriction site on the upstream side and the NcoI restriction site on the downstream side. The -103 to +51 fragment was engineered to contain the NcoI site upstream and the HindIII site downstream. Following PCR amplification each fragment was double-digested with the appropriate restriction enzymes (NheI and NcoI or NcoI and HindIII). A three way ligation with the two PCR-generated fragments and pGL3-E1bTATA, double-digested with NheI and HindIII, then was performed, replacing the *bax* sequence from -113 to -104 with the NcoI restriction site. The generation of pBaxΔ-103/-93, pBaxΔ-92/-83, and pBaxΔ-113/-93 was accomplished as above with pBaxΔ-113/-104 but using PCR-generated fragments corresponding to -315 to -104 and -92 to +51, -315 to -93 and -82 to +51, and -315 to -114 and -92 to +51 respectively. The expression plasmid pCMV-p53^{wt}, originally referred to as pC53-SN3 (35), encodes the wild-type human p53 protein under the control of the cytomegalovirus promoter. The expression plasmid pPacSp1 contains the 2.1 kb XhoI restriction fragment of Sp1 cloned downstream of the

Actin 5C promoter (36). pPacU was generated by removing the 2.1 kb XhoI fragment from pPacSp1.

Transfections

Saos-2 cells were transfected using Lipofectamine Plus Reagent (GibcoBRL, Life Technologies). 2 x 10^5 cells were seeded into 35-mm plates. Cells were transfected 24 h later according to the manufacturer's instructions. Cellular lysates were prepared 24 h post-transfection, total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a commercially available kit (Promega) and a TD-20e Luminometer (Turner). *Drosophila* SL2 cells were transfected using Cellfectin (GibcoBRL, Life Technologies). 60-mm dishes were seeded with 2 x 10^6 cells in Schneider's *Drosophil*a media containing 10% heat-inactivated fetal bovine serum, but no penicillin or streptomycin. The DNA to be transfected was added to 500 μl of serum free media containing 8 μl of Cellfectin reagent, mixed gently, and incubated at room temperature for 20 min. This mixture then was added directly to the cells. 48 h post transfection cells were lysed by sonication (6 x 20 sec pulse). Total protein and luciferase activity was determined as above.

HeLa cell nuclear extraction

Unless otherwise stated, all procedures were conducted at 4°C. HeLa S3 cells were obtained as a packed cell pellet from the National Cell Culture Center (Minneapolis, MN). Cell pellets were resuspended in 5 volumes of Buffer A (10mM HEPES pH7.6, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT) and incubated on ice for 10 min. Cells then were centrifuged at 500xg for 12 min. The supernatant was removed and the pellet was resuspended in two packed-cell volumes of Buffer A. Cells were homogenized 10-times in a Dounce homogenizer with pestle A (tight). The resulting solution was centrifuges at 430xg for 10 min to pellet the nuclei. The supernatant was decanted and the pellet was recentrifuged at 24,000xg for 20 min. The supernatant again was removed. The pellet was resuspended in 3 ml of Buffer C (20mM HEPES pH7.6, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT) per 10^9 cells. The solution was homogenized 10-times with pestle B (loose). The resulting solution was transferred to a beaker and stirred for 30 min on ice. The solution then was centrifuged at 24,000xg for 30 min. The resulting nuclear extract was dialyzed against Buffer D (20mM HEPES pH7.6, 20% glycerol, 0.1M KCl, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM DTT) for 5 h. The extract was clarified by centrifugation at 24,000xg for 20 min. Nuclear extracts were aliquoted, frozen in a dry-ice/ethanol bath, and stored at -70°C.

Electrophoretic Mobility Shift Assay

Production of baculovirus-infected Sf9 cell extracts and purification of recombinant human p53 protein was done as previously described (34). Purified p53 protein, extract from Sf9 cells expressing recombinant human Sp1 protein, or HeLa cell nuclear extract was incubated with 3 ng of radiolabeled double-stranded oligonucleotide and antibody (Sp1 PEP-2X, p300 N-15X, and CBP 451X, Santa Cruz Biotechnology), where appropriate, in a total volume of 30 μl of DNA-binding buffer (20mM HEPES pH7.5, 83mM NaCl, 0.1mM EDTA, 12% glycerol, 2mM $MgCl_2$, 2mM spermidine, 0.7mM DTT, and 25 μg/ml poly d(I-C)) for 20 min at room temperature. Samples were loaded on a native 4% acrylamide gel in 0.5X TBE and electrophoresed at 4°C at 225V for 2 h. The gel was dried and exposed to Kodak XAR film using an intensifying screen at -70°C. Phosphorimaging and densitometry data were collected with a Personal Molecular Imager FX and a GS-710 Calibrated Imaging Densitometer (Bio-Rad), and analyzed with Quantity One software (Bio-Rad).

Results

All three potential p53 half-sites are required for the p53-dependent transcriptional activation of the human *bax* promoter

Previously it was demonstrated that in isolation the p53 response element from the human *bax* promoter required sequences from three adjacent half-sites to confer p53-dependent transcriptional activation on a minimal promoter (37). To confirm the requirement of all three half-sites in the context of the *bax* promoter, luciferase reporter plasmids with various deletions in the *bax* promoter, both in and around the p53 response element, were cotransfected with either pCMV or a wild-type p53 expression vector into the p53-negative osteosarcoma Saos-2 cell line (Figure 1). The previously characterized p53 response element of the *bax* promoter is contained within the sequence from -113 to -83 from the start-site of transcription. There was no significant difference between the p53-dependent transactivation of either a reporter construct lacking sequences 5' to the p53 response element (pBax-127/+51) or the full-length promoter construct (pBax-315/+51)(Figure 1A). Deletion of a larger fragment, including the p53 response element (pBax-76/+51), produced a reporter construct that was unresponsive to wild-type p53 (Figure 1A). Further, targeted deletion of the promoter region containing the p53 response element (pBaxΔ-126/-77) also produced a reporter plasmid that was unresponsive to wild-type p53 (Figure 1A). These results show that -113 to -83 is the

only region, within the 366 bp promoter fragment investigated, that affects the ability of p53 to activate transcription.

The region from -113 to -83 contains three potential p53 half-sites (represented in Figure 1B as the light grey, white, and dark grey boxes). The role of each of these half-sites in the p53-dependent activaiton of the *bax* promoter was examined. Removal of the first half-site from -113 to -104 (pBaxΔ-113/-104) significantly reduced the ability of p53 to activate transcription through this promoter (Figure 1B, compare 63-fold with pBax-315/+51 to 7-fold with pBaxΔ-113/-104), while removal of the second (pBaxΔ-103/-93) or the third half-site (pBaxΔ-92/-83) completely abolished the ability of p53 to transcriptionally activate the promoter (Figure 1B). Consistent with the above results, removal of the first and second half-sites in combination (pBaxΔ-113/-93) also abolished the ability of p53 to transcriptionally activate the promoter (Figure 1B). These results demonstrate that, as was observed with the isolated response element (37), p53 requires sequences from all three potential half-sites to mediate transcriptional activation of the *bax* promoter.

The first two potential p53 half-sites constitute a *bona fide* p53 response element

Each of the three potential p53 half-sites located in the *bax* promoter from -113 to -83 closely resemble the consensus sequence of 5'-RRRCWWGYYY-3' (represented in

Figure 2 by the light grey, white, and dark grey boxes). The first, located at -113 to -104, deviates from the consensus at two bases (-113 and -104). The second half-site matches the consensus sequence at all 20 base pairs and is located at -102 to -93. The third half-site is located at -92 to -83 and deviates from the consensus at three bases (-84, -85, and -88)(see Figure 2). These three half-sites can combine in different ways to produce a total of three possible p53 complete binding sites (half-sites 1 and 2, 2 and 3, and 1 and 3). Previous studies demonstrated that in electrophoretic mobility shift assays (EMSA) double-stranded oligonucleotides representing both -113 to -93 (half-sites 1 and 2) and -102 to -83 (half-sites 2 and 3) are capable of binding p53 in a sequence-specific manner with similar affinities. When cloned upstream of the adenovirus *E1b* minimal promoter in the pTATA luciferase reporter plasmid, however, the combination of the first and second half-sites (-113 to -93) is unable to mediate p53-dependent transcriptional activation (37). To further examine the ability of p53 to interact with this sequence in cells, the -113 to -93 sequence was multimerized (as three copies) and cloned into the pTATA luciferase reporter plasmid. This reporter plasmid was cotransfected with either pCMV or a wild-type p53 expression vector in the Saos-2 cell line (Figure 2). These three copies of this p53 binding site were capable of mediating a significant degree of activation in response to p53 (Figure 2, compare 4-fold with pTATA-113/-93 to 142-fold with pTATA$(-113/-93)_3$), demonstrating that the sequence from -113 to -93 is indeed a *bona fide* p53 response element capable of both binding p53 in a sequence-specific

manner *in vitro* and mediating p53-dependent transcriptional activation in cells. Confirming previous results, p53 was able to activate transcription through the second and third half-sites (-102 to -83), but this activation was significantly reduced as compared to that mediated by all three half-sites combined (Figure 2, compare 44-fold with pTATA-102/-83 and 153-fold with pTATA-113/-83). To test the ability of half-sites one and three to mediate p53-dependent transcriptional activation, a synthetic oligonucleotide corresponding to -113 to -83 of the *bax* promoter, with -102 to -93 scrambled to remove any contribution of the second half-site, was cloned into the pTATA reporter plasmid. This construct failed to be activated by p53 (Figure 2, pTATA-113/-83(sc-102/-93)). The third half-site in isolation (-92 to -83) also failed to mediate p53-dependent transcriptional activation (Figure 2, pTATA-92/-83).

Sp1 binds with sequence-specificity to and activates transcription through the p53 response element from the human *bax* promoter

We previously reported the identification of a nuclear factor, termed Binder of Bax 1 (BoB1), that interacts with sequence specificity with the same region of the human *bax* promoter that is required for p53-dependent transcriptional activation (37). These previous studies demonstrated that this factor binds to sequences within the region of -102 to -83. Analysis of this region using a MatInspector search of the TRANSFAC database (38,39) showed that it contains sequence that potentially could bind the

transcription factor Sp1. To test this, a synthetic oligonucleotide corresponding to -102 to -83 of the *bax* promoter was used as a radiolabeled probe in an EMSA with HeLa cell nuclear extract (Figure 3). As previously reported for Saos-2, HeLa cell nuclear extract contains a factor that demonstrated marked sequence specificity for the labeled *bax* probe. This factor was successfully competed by increasing amounts of unlabeled probe (Figure 3, lanes 7-9) as well as by increasing amounts of oligonucleotide corresponding to the DNA-binding consensus sequence of Sp1 (Figure 3, lanes 13-15). This binding was specific, as an oligonucleotide corresponding to the 5' p53 response element from the human *p21* promoter failed to compete for binding (Figure 3, lanes 10-12). In addition, this factor was successfully bound by an anti-Sp1 antibody, as demonstrated by a "super-shifted" complex (Figure 3, lanes 2 and 3), while a control anti-p300 antibody failed to bind the factor (Figure 3, lanes 4 and 5). Together, these data demonstrate that Sp1 can bind a portion of the p53 response element from the human *bax* promoter in a sequence-specific manner.

To further delineate the sequences important for Sp1 binding, oligonucleotides were synthesized that replaced portions of the *bax* sequence with corresponding sequence from the *p21*-5' p53 response element. The sequence from -102 to -83 in the *bax* promoter contains two p53 half-sites (-102 to -93 and -92 to -83), and the p21-5' element also consists of two p53 half-sites. Hybrid oligonucleotides were synthesized in which

the first of the two half-sites in the *bax* element was combined with the second half-site of the *p21*-5' element and vice versa. The oligonucleotide corresponding to -102 to -83 of the *bax* promoter again was used as a radiolabeled probe with HeLa nuclear extract in an EMSA (Figure 4). Competitions, using unlabeled probe as well as the oligonucleotides corresponding to the *p21*-5' element and the two hybrid elements, were conducted. Sp1 bound the radiolabeled probe (Figure 4, lane 1) and was recognized by an anti-Sp1 antibody (Figure 4, lane 2) but not by a control anti-CBP antibody (Figure 4, lane 3). Both unlabeled probe and the Sp1 DNA-binding consensus site oligonucleotide effectively competed for Sp1 binding (Figure 4, lanes 4-5 and 12-13 respectively), while the *p21*-5' element did not (Figure 4, lanes 10-11). Consistent with the notion that Sp1 binds DNA through GC-box regions, the hybrid oligonucleotide in which the first half-site is derived from the *p21* sequence and the second half-site from the *bax* sequence (-92 to -83: 5'-GGGCGTGGGC-3') effectively competed for Sp1 binding (Figure 4, lanes 8-9), while the other hybrid oligonucleotide which replaces this GC-rich region with sequence from the *p21*-5' element demonstrated a significantly reduced affinity for Sp1 binding (Figure 4, lanes 6-7). These data indicate that Sp1 binds to sequence within -92 to -83 of the *bax* promoter.

To determine whether or not Sp1 can interact with this element in cells, a pTATA luciferase reporter plasmid containing -113 to -77 of the human *bax* promoter was

cotransfected with increasing amounts of a Sp1 expression vector into the Sp1-deficient *Drosophila* SL2 cell line (Figure 5). Expression of Sp1 successfully activated transcription of this reporter, yet failed to activate transcription of a control plasmid containing the 5' p53 response element of the *p21* promoter (Figure 5). Consistent with the *in vitro* EMSA results, this confirms that Sp1 is capable of activating transcription through the p53 response element of the human *bax* promoter.

The ability of Sp1 to bind the p53 response element of the *bax* promoter *in vitro* correlates with the ability of p53 to activate transcription through this element in cells

To explore the significance of the Sp1 binding site to the ability of p53 to activate transcription through the *bax* promoter, nucleotide substitutions were identified that differentially affected the ability of p53 to activate transcription through its response element in the *bax* promoter (-113 to -83). Two mutated forms of the p53 response element from the *bax* promoter, in which the indicated guanine bases were replaced with adenines (Figure 6A, GG-92/-91AA and GG-85/-84AA), were cloned into the pTATA luciferase reporter plasmid. In cotransfection assays with a wild-type p53 expression vector in the Saos-2 cell line, substitution of bases -92 and -91 completely abolished the ability of p53 to activate transcription through this element (Figure 6A, compare -113/-83 to GG-92/-91AA), while substitution of bases -85 and -84 did not (Figure 6A). As

observed in Figures 1 and 2, removal of the third potential half-site (-92 to -83) inhibited the ability of p53 to mediate transcriptional activation through this element (Figure 6A, compare -113/-83 and -113/-93), demonstrating the requirement for this Sp1-binding sequence in the p53-dependent transcriptional activation of this element.

Both of these mutant sequences were assayed for their ability to bind purified p53 in an EMSA. An oligonucleotide corresponding to -113 to -77 of the *bax* promoter was used as radiolabeled probe with purified p53 in an EMSA (Figure 6B). Competitions were performed with increasing amounts of an oligonucleotide corresponding to -113 to -83 of the *bax* promoter and the two mutant oligonucleotides. When compared to the wild-type oligonucleotide both mutant oligonucleotides displayed a slightly decreased affinity for p53 (Figure 6B, compare lanes 2-4 with lanes 5-7 and 8-10; Figure 6C). Compared to one another, however, both mutant oligonucleotides demonstrated a comparable affinity for p53 (Figure 6A and B), suggesting that the differences in p53-dependent transcriptional activation observed in Figure 6A are not due to differences in the affinity of p53 for the two sequences. In contrast, the abilities of the two mutant sequences to bind Sp1 differed (Figure 6D and E). An oligonucleotide corresponding to -113 to -77 of the *bax* promoter was used as radiolabeled probe with extract from Sf9 cells expressing recombinant human Sp1 protein in an EMSA (Figure 6D). Sp1 bound the probe and was recognized by an anti-Sp1 antibody (Figure 6D, lanes 1-2). Sp1 binding

was successfully competed by unlabeled Bax-113/-83 oligonucleotide as well as by the GG-85/-84AA mutated oligonucleotide (Figure 6D, lanes 3-4 and 9-11 respectively; Figure 6E). The GG-92/-91AA mutant, however, demonstrated a significant decrease in affinity for Sp1 (Figure 6D, compare lanes 3-5 and 9-11 to lanes 6-8; Figure 6E).

The results with the GG-85/-84AA mutant presented in Figure 6 suggest that not all of the bases contained within the third potential half-site of the p53 response element are required for p53-dependent transcriptional activation. To identify the minimal sequence elements required to mediate p53-dependent transactivation, a series of oligonucleotides was synthesized in which each of the ten bases of the third potential half-site (-92 to -83) was individually replaced. These mutant oligonucleotides then were cloned into the pTATA luciferase reporter plasmid and tested for their responsiveness to p53 in a cotransfection assay in the Saos-2 cell line (Figure 7). Consistent with the results in Figure 6A, substitution of the bases at either -85 or -84 did not inhibit the ability of p53 to activate transcription through this element (Figure 7, G-85T and G-84T). Further, substitution of -86 and -83 also failed to significantly affect the ability of p53 to activate transcription (Figure 7, compare -113/-83 to G-86T and C-83A). Substitution of the base at -87, however, significantly reduced the ability of p53 to activate transcription through this element (Figure 7, compare -113/-83 to T-87G). Together, these results

suggest that the minimal response element consists of sequence from -113 to -87, with -86 to -83 being dispensable for p53-dependent transactivation.

To confirm that the bases from -86 to -83 are not required for p53-dependent transcriptional activation, two additional mutant oligonucleotides were synthesized. The first mutant was generated by replacing all ten nucleotides from -92 to -83 (Figure 8A, sc-92/-83). The four bases from -86 to -83 were substituted as indicated to generate the second mutant oligonucleotide (Figure 8A, sc-86/-83). Each oligonucleotide was cloned into the pTATA vector, and tested for its responsiveness to p53 in a cotransfection assay (Figure 8A). As observed with the reporter plasmid in which the sequence from -92 to -83 is removed entirely (pTATA-113/-93), the first mutant, in which all ten bases of the third potential half-site (-92 to -83) are replaced, showed little to no response to p53 (Figure 8A, compare pTATA-113/-93 to pTATA-113/-83 and pTATAsc-92/-83). In contrast, the second mutant, in which only the last four bases of the element (-86 to -83) are replaced, was efficiently activated by p53 (Figure 8A, compare 312-fold with pTATA-113/-83 to 323-fold with pTATAsc-86/-83). This result demonstrates that the minimal p53 response element in the *bax* promoter consists of sequence from -113 to -87. In an EMSA both mutants displayed a decreased affinity for p53 as compared to the wild-type sequence (Figure 8B, compare lanes 2-4 to lanes 8-10 and 11-13; Figure 8C). When compared to each other, there was no significant difference in the affinity of p53 for the

two mutant sequences (Figure 8B, compare lanes 8-10 to 11-13; Figure 8C). This suggests that the differences in transcriptional activation observed in Figure 8A cannot be explained by differences in p53 affinities. Further, the oligonucleotide corresponding to -113 to -93 displayed a similar affinity for p53 as the two mutant oligonucleotides (Figure 8B, compare lanes 5-7 to lanes 8-10 and 11-13; Figure 8C) consistent with the idea that, in the case of the two mutants, p53 is interacting with the first and the second half-sites only. The sc-86/-83 mutant oligonucleotide efficiently competed for Sp1 binding in an EMSA (Figure 8D, compare lanes 2-4 to lanes 8-10; Figure 8E), while the ability of the sc-92/-83 mutant to bind Sp1 was significantly reduced compared to the wild-type sequence (Figure 8D, compare lanes 2-4 to lanes 5-7; Figure 8E), further strengthening the correlation between Sp1 binding *in vitro* and p53 activation in cells.

Discussion

The data presented in this report demonstrate that the minimum p53 response element in the *bax* promoter consists of the sequence from -113 to -87 from the start site of transcription. This sequence contains a p53 binding site (-113 to -93) that can function as a *bona fide* response element as demonstrated by its ability when multimerized to confer p53-dependent transcriptional activation on a minimal promoter (Figure 2). Immediately adjacent to this p53 binding site are six base pairs that are GC-rich in nature (-92 to -87: 5'-GGGCGT-3'). These six bases are required for p53-dependent transcriptional activation as deletion or mutation of this region in the context of either the promoter or the isolated response element completely abrogates the ability of p53 to activate transcription through this sequence (Figures 1B, 2, 6A, and 8A). The addition of these bases to the -113/-93 sequence appears to have little effect on the affinity of p53 for this sequence (Figure 8B and C), consistent with a model in which these six bases function to recruit a co-activator as opposed to simply enhancing p53 binding. Further, these six base pairs mediate sequence-specific binding to the Sp1 transcription factor (Figures 3, 4, 6D, and 8D), and a positive correlation is seen between the ability of Sp1 to bind this element *in vitro* and the ability of p53 to mediate transcriptional activation through its response element in cells (Figures 6 and 8). In addition, the results with electophoretic mobility shift assays with the GG-92/-91AA mutant oligonucleotide (Figure 6B) are not

consistent with the published p53 DNA-binding consensus sequence of (RRRCWWGYYY)2 (18,19). This consensus allows for a purine in the first three positions of each half-site. The GG-92/-91AA mutant contains a conservative substitution of purines (adenines) for purines (guanines), and, as such, does not represent a substantive change in terms of the p53 DNA-binding consensus sequence. This substitution, however, did produce a significant decrease in the ability of p53 to bind to this oligonucleotide *in vitro* (Figure 6B), suggesting that, in these limited circumstances, the p53 DNA-binding sequence involves greater specificity than implied by the consensus.

Previous studies have suggested a connection between p53 and Sp1. The two proteins physically interact under certain circumstances (40-42), and, transcriptionally, p53 and Sp1 have been shown to function in a cooperative manner in some settings and an antagonistic manner in others (41,43,44). In addition to p53, Sp1 has been found to synergize with other transcription factors, including YY1 and SREBP (45-47). Studies with the Sp-family of transcription factors, however, are complicated by the fact that there are at least 16 mammalian members of this family. Due to marked conservation in the DNA-binding domain, many of these family members have similar if not identical *in vitro* DNA-binding characteristics (48,49). Originally, this led to the misclassification of many GC-boxes solely as Sp1-binding sites because of the ubiquitous nature of Sp1 and

the fact that it was the first family member cloned. Given this, the possibility exists that the true *in vivo* cofactor required for the p53-dependent transactivation of the *bax* promoter is a Sp1-related family member that is obscured in *in vitro* assays by the sheer abundance of Sp1 in nuclear extracts from tissue culture cells. Consistent with this, antibodies used in a super-shift EMSA identified other Sp-family members as minor components of the Sp1-DNA complex (E. C. Thornborrow and J. J. Manfredi, unpublished data). Further, cotransfection assays in the Sp1-deficient *Drosophila* SL2 cell line failed to demonstrate cooperation between Sp1 and p53 in transcriptionally activating the p53 response element of the *bax* promoter (E. C. Thornborrow and J. J. Manfredi, unpublished data). The *Drosophil*a assays, however, are difficult to interpret as the ability of p53 alone to activate transcription through a control plasmid was significantly impaired in the SL2 cell line. Complicating interpretation of the results in the *Drosophila* system is the recent identification of a *Drosophila* p53 homolog (50,51) which may affect the ability of transiently expressed human p53 to function properly in this system.

Regardless of whether the cofactor required for the p53-dependent transactivation of the *bax* promoter is Sp1 or a related family member, the requirement of this cooperating protein suggests a model for the observed cell-type and tumor-type specific regulation of the *bax* gene by wild-type p53 (Figure 9). In this model, cells that are

permissive to p53-dependent upregulation of the *bax* gene express both p53 and the cofactor and these proteins function together to transcriptionally activate the gene. In those cells that fail to show p53-dependent *bax* expression one can propose three possible mechanisms to explain the apparent failure of wild-type p53 to activate the *bax* gene (Figure 9). First, the required cofactor may be absent, either due to mutation or due to cell-type specific limitations on its expression. Second, this factor may be inactivated by post-translational modification. Finally, another factor that cannot cooperate with p53 may compete with the cofactor for binding to its site in the *bax* promoter. Data with the Sp-family of transcription factors support each of these possibilities. While several of the Sp-family members, like Sp1, are ubiquitously expressed, other members of the family display high degrees of tissue specificity (48,49). Even the ubiquitously expressed family members fluctuate in levels under particular cellular conditions (52-55). Sp1 mRNA, for example, varies up to a 100-fold depending on the cell type and developmental stage of the mouse (56). Consistent with a model of post-translational modification, certain Sp-family members, including Sp1 and EKLF, are phosphorylated, glycosylated, and acetylated (57-59). Finally, given the high level of conservation in the DNA-binding domain of the Sp-family of transcription factors, it is not surprising that DNA-binding competition can be observed between various members of this family. In certain cases, including Sp1/Sp3, BTEB1/AP-2rep, and BKLF/EKLF, this competition has ramifications on gene expression (60-62). In each case, transcriptional activation by one

family member is repressed by the other member by competing for the same DNA-binding site. The data in this report, in combination with the previous studies of the Sp-family of transcription factors, support a model in which the regulation of a required cofactor controls cell-type specific p53-dependent expression of the *bax* gene.

The ability of the proapoptotic Bax to function as a tumor suppressor protein has been substantiated by several studies. In certain mouse models, Bax has been shown to be an important mediator of p53-dependent apoptosis and a suppressor of oncogenic transformation, with loss of *bax* leading to accelerated rates of tumor growth, increased tumor numbers, larger tumor mass, and decreased survival rates (63,64). A significant correlation between decreased Bax expression and both a corresponding resistance to apoptotic stimuli, as well as, a shorter survival period also has been observed in a number of human tumor types, including breast, ovarian, pancreatic, colorectal, and non-Hodgkin lymphoma (65-69). In addition, in colon and gastric cancers of the microsatellite mutator phenotype mutational inactivation of the *bax* gene has been shown to confer a strong survival advantage during tumor clonal evolution (70). Complimenting these data are observations showing that overexpression of the Bax protein in certain tumor cell lines both sensitizes these cells to chemotherapy- and radiation-induced apoptosis and reduces their ability to form tumors in SCID mice (71-73). Together, these results strongly support a tumor suppressor role for the Bax protein.

An important regulator of the *bax* gene is the tumor suppressor protein p53. Several reports have demonstrated the significance of the p53-Bax pathway in tumor suppression. Both the identification of tumor derived p53 mutants that selectively fail to activate transcription through the *bax* promoter and subsequently fail to induce apoptosis (29-32), as well as the TgT121 transgenic studies that demonstrate that *bax* is an obligatory downstream effector of p53 in the suppression of choroid plexus tumor growth (33) suggest that the ability of p53 to activate transcription through the *bax* promoter is important to the tumor suppressor function of p53. Further, the resistance of certain tumor cell lines to radiation therapy is associated with a failure of wild-type p53 to induce *bax* expression (28,74), and certain human tumors have been identified that are genetically wild-type for both *p53* and *bax*, and, yet, fail to express significant levels of Bax protein (75). Thus, a complete understanding of the transcriptional regulation of the *bax* gene by the tumor suppressor p53 may provide important information concerning both the molecular origins of cancer as well as the development of tumor resistance to certain cancer treatments.

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Figure Legends

Figure 1. All three potential p53 half-sites are required for the p53-dependent transcriptional activation of the human *bax* promoter.

(A) and (B) Saos-2 cells were transfected as described under Experimental Procedures with 1 μg of the indicated pBax reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey bars). 24 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental Procedures. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} as compared with pCMV. (A) The previously identified p53 response element is indicated by the dark grey box at -113 to -83. (B) The three potential p53 half-sites are represented by the light grey (-113 to -104), white (-102 to -93), and dark grey (-92 to -83) boxes.

Figure 2. The first two potential p53 half-sites constitute a *bona fide* p53 response element.

Saos-2 cells were transfected as described under Experimental Procedures with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey bars). 24 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental Procedures. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} as compared with pCMV. The sequence of the *bax* promoter from -113 to -83 is given at the top of the figure. Potential p53 quarter-sites are indicated by the solid bars above and below the sequence. Bases that deviate from the p53 DNA-binding consensus sequence are indicated by asterisks. The three potential half-sites are indicated by the brackets labeled 1, 2, and 3 respectively, and are represented graphically as the light grey, white, and dark grey boxes respectively. The vertical arrow above the *bax* sequence indicates the one base pair insert between the first and second half-sites.

Figure 3. Sp1 binds with sequence specificity to the p53 response element from the human *bax* promoter.

An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -102/-83 sequence from the human *bax* promoter as radiolabeled

probe. 8 μg of HeLa cell nuclear extract was incubated with 3 ng of the probe alone (lane 1 and 6), in the presence of 2 or 4 μl of anti-Sp1 antibody (lanes 2 and 3 respectively), 2 or 4 μl of anti-p300 antibody (lanes 4 and 5 respectively), a 100-, 200-, or 300-fold molar excess of either the unlabeled Bax-102/-83 oligonucleotide (lanes 7-9) or p21-5' oligonucleotide (lanes 10-12), or a 10-, 20-, or 30-fold molar excess of the unlabeled Sp1 consensus oligonucleotide (lanes 13-15). The arrows indicate the positions of the Sp1-DNA complex and the super-shifted complex containing antibody, Sp1, and DNA.

Figure 4. The Sp1 binding site is localized to a GC-rich region of the p53 response element.

(A) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -102/-83 sequence from the human *bax* promoter as radiolabeled probe. 8 μg of HeLa cell nuclear extract was incubated with 3 ng of the probe alone (lane 1), in the presence of 4 μl of anti-Sp1 antibody (lane 2), 4 μl of anti-CBP antibody (lane 3), a 100- or 200-fold molar excess of either the unlabeled Bax-102/-83 oligonucleotide (lanes 4 and 5), p21-5' oligonucleotide (lanes 10 and 11), Bax/p21-5' hybrid oligonucleotide (lanes 6 and 7) or p21-5'/Bax hybrid oligonucleotide (lanes 8 and 9), or a 10- or 20-fold molar excess of the unlabeled Sp1 Consensus oligonucleotide (lanes 12 and 13). The arrows indicate the positions of the Sp1-DNA and the super-shifted

antibody-Sp1-DNA complexes. (B) The sequences of the human *bax* promoter from -102 to -83 from the start site of transcription (grey boxes) and the human *p21* promoter from -2281 to -2262 from the start site of transcription (white boxes; corresponding to the p21-5' oligonucleotide) are shown. Each sequence is divided into two with the first half indicated as A and the second half indicated as B. Oligonucleotides in (A) are represented graphically according to this color and letter scheme. For example, the Bax/p21-5' hybrid oligonucleotide which corresponds to the first half of the *bax* sequence followed by the second half of the *p21* sequence is indicated by a grey box labeled A followed by a white box labeled B.

Figure 5. Sp1 can activate transcription through the p53 response element of the human *bax* promoter.

Drosophila SL2 cells were transfected as described under Experimental Procedures with 2 μg of the indicated pTATA reporter constructs in the presence of 0, 300, 600, or 900 ng of pPacSp1. Appropriate amounts of the vector pPacU were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.9 μg/sample. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental Procedures. The indicated values are the average of four

independent experiments expressed as the fold activation for each reporter plasmid with pPacSp1 as compared with pPacU. Error bars correspond to one standard deviation.

Figure 6. A mutant element that fails to bind Sp1 *in vitro* also fails to confer p53-dependent transcriptional activation in cells.

(A) Saos-2 cells were transfected as described under Experimental Procedures with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey bars). 24 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental Procedures. The indicated values are the average of five independent experiments each performed in duplicate. Error bars correspond to one standard deviation. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in grey with the corresponding mutations indicated above. (B) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-77 sequence from the human *bax* promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled Bax-113/-83 oligonucleotide (lanes 2-4), the BaxGG-92/-91AA oligonucleotide (lanes 5-7), or the BaxGG-85/-84AA oligonucleotide (lanes 8-10). The arrow indicates the position of the

p53-DNA complex. The vertical bar between lanes 4 and 5 represents the removal of irrelevant lanes from the gel. (C) Bands were quantitated by densitometry. (D) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-77 sequence from the human *bax* promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 2), in the presence of anti-Sp1 antibody (lane 1), or in the presence of a 50-, 100-, or 200-fold molar excess of either the unlabeled Bax-113/-83 oligonucleotide (lanes 3-5), the BaxGG-92/-91AA oligonucleotide (lanes 6-8), or the BaxGG-85/-84AA oligonucleotide (lanes 9-11). The arrow indicates the position of the Sp1-DNA complex and the asterisk indicates the position of the super-shifted Ab-Sp1-DNA complex. The vertical bar between lanes 5 and 6 represents the removal of irrelevant lanes from the gel. (E) Bands were quantitated by densitometry.

Figure 7. Mutational analysis shows that the *bax* promoter sequence from -86 to -83 is not required for p53-dependent transcriptional activation.

Saos-2 cells were transfected as described under Experimental Procedures with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey bars). 24 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental

Procedures. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in grey with the corresponding mutations indicated above.

Figure 8. The minimal element from the *bax* promoter that confers p53-dependent transcriptional activation consists of a single p53 binding site and an adjacent Sp1 binding site.

(A) Saos-2 cells were transfected as described under Experimental Procedures with 1 μg of the indicated pTATA reporter constructs in the presence of either 10 ng of pCMV (white bars) or 10 ng of pCMV-p53^{wt} (grey bars). 24 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under Experimental Procedures. The indicated values are the average of three independent experiments each performed in duplicate. Error bars correspond to one standard deviation. The numbers above each bar indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} as compared with pCMV. The GC-rich region that binds Sp1 is shown by the boxed sequence. Bases in the wild-type sequence that were mutated are shown in grey with the corresponding mutations indicated above. (B) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-83

sequence from the human *bax* promoter as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 500-, 1000-, or 1500-fold molar excess of either the unlabeled Bax-113/-83 oligonucleotide (lanes 2-4), the Bax-113/-93 oligonucleotide, the Bax sc-92/-83 oligonucleotide (lanes 5-7), or the Bax sc-86/-83 oligonucleotide (lanes 8-10). The arrow indicates the position of the p53-DNA complex. (C) Bands were quantitated by phosphorimaging. (D) An electrophoretic mobility shift assay was performed using an oligonucleotide corresponding to the -113/-83 sequence from the human *bax* promoter as radiolabeled probe. Extract from Sf9 cells expressing human recombinant Sp1 protein was incubated with 3 ng of the probe alone (lane 1) or in the presence of a 10-, 50-, or 100-fold molar excess of either the unlabeled Bax-113/-83 oligonucleotide (lanes 2-4), the Bax sc-92/-83 oligonucleotide (lanes 5-7), or the Bax sc-86/-83 oligonucleotide (lanes 8-10). The arrow indicates the position of the Sp1-DNA complex. (E) Bands were quantitated by phosphorimaging.

Figure 9. Model for the cell-type specific regulation of the *bax* promoter by the tumor suppressor protein p53.

(A) In cells that are permissive to p53-dependent transcriptional activation of the *bax* gene, p53 and the required cofactor cooperate to mediate activation. In cells that do not support the p53-Bax pathway, three possible mechanisms may explain the apparent

failure of wild-type p53 to activate the *bax* gene. (B) The cofactor may be absent due to mutation or to cell-type specific limitations on its expression. (C) The cofactor may be inactivated by post-translational modification, such as phosphorylation "P", glycosylation "G", or acetylation "A". (D) Another factor that cannot cooperate with p53 may compete with the required cofactor for binding to its site in the *bax* promoter. The p53 binding site (-113 to -93) is represented by the black box. The Sp1 binding site (-93 to -87) is represented by the white box. p53, the required cofactor, and the inhibitory factor are represented by the grey circle, the dotted oval, and the cross-hatched triangle respectively.

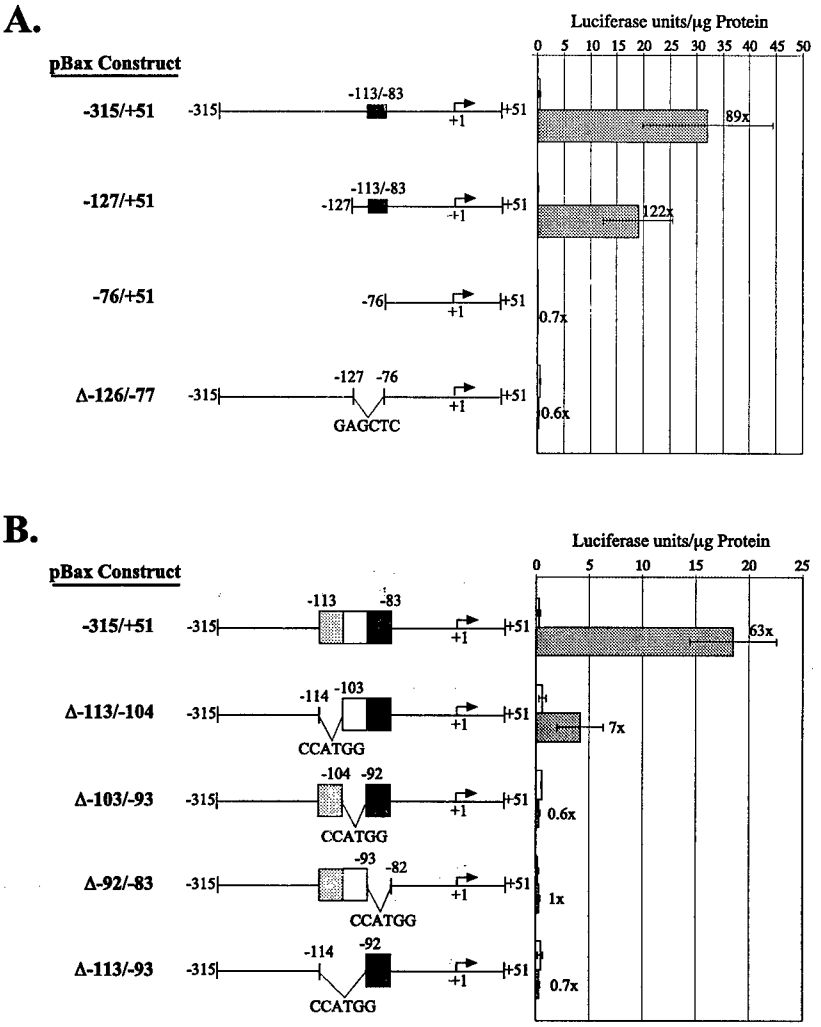

A.
pBax Construct
Luciferase units/μg Protein
0 5 10 15 20 25 30 35 40 45 50
-315/+51
-315
-113/-83
+1
+51
89x
-127/+51
-127
-113/-83
122x
-76/+51
-76
0.7x
Δ-126/-77
-315
-127 -76
GAGCTC
0.6x
B.
pBax Construct
Luciferase units/μg Protein
0 5 10 15 20 25
-315/+51
-113 -83
63x
Δ-113/-104
-114 -103
CCATGG
7x
Δ-103/-93
-104 -92
CCATGG
0.6x
Δ-92/-83
-93 -82
CCATGG
1x
Δ-113/-93
-114 -92
CCATGG
0.7x

Fig. 1

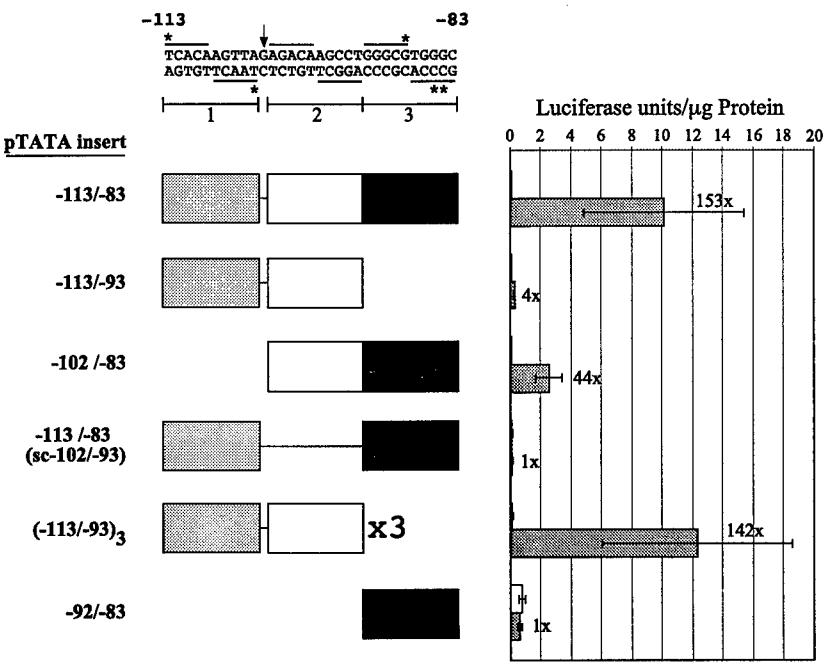

-113
-83
TCACAAGTTAGAGACAAGCCTGGGCGTGGGC
AGTGTTCAATCTCTGTTCGGACCCGCACCCG
1
2
3
pTATA insert
-113/-83
-113/-93
-102 /-83
-113 /-83
(sc-102/-93)
(-113/-93)3
x3
-92/-83
Luciferase units/µg Protein
0 2 4 6 8 10 12 14 16 18 20
153x
4x
44x
1x
142x
1x

Fig. 2

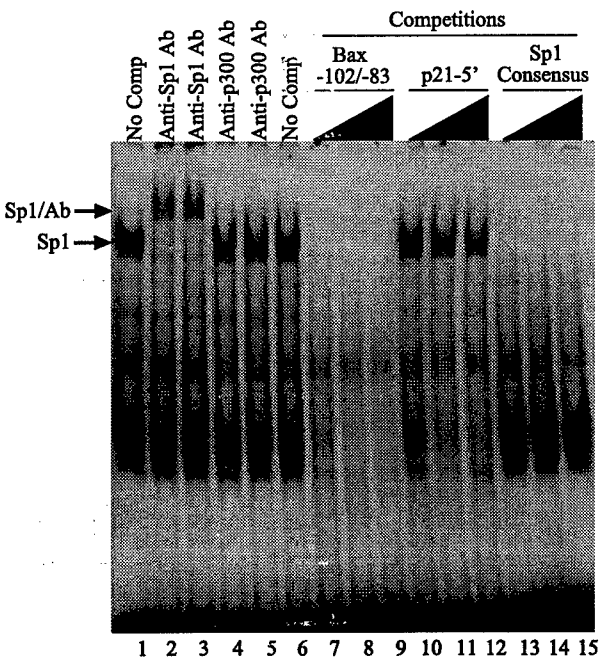
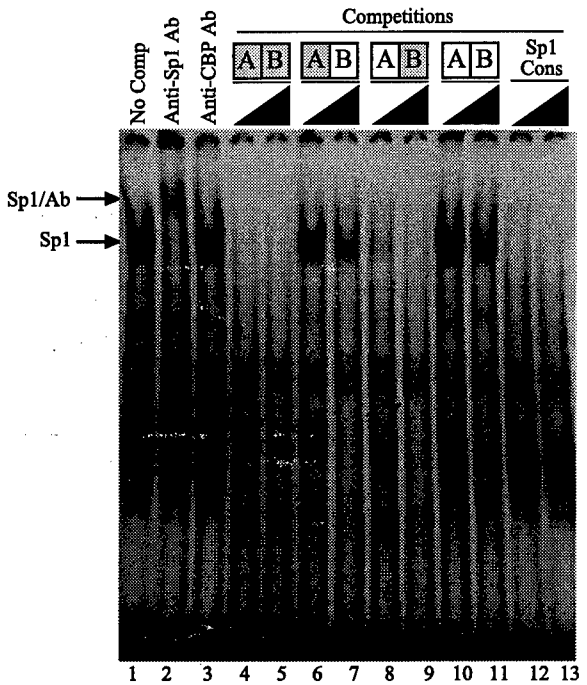

Competitions
Bax -102/-83
p21-5'
Sp1 Consensus
No Comp
Anti-Sp1 Ab
Anti-Sp1 Ab
Anti-p300 Ab
Anti-p300 Ab
No Comp
Sp1/Ab
Sp1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 3

A.


Competitions
No Comp
Anti-Sp1 Ab
Anti-CBP Ab
A B
A B
A B
A B
Sp1 Cons
Sp1/Ab
Sp1
1 2 3 4 5 6 7 8 9 10 11 12 13

B.


A
B
Bax-102/-83
AGACAAGCCTGGGCGTGGGC
TCTGTTCGGACCCGCACCCG
A
B
p21-5'
GAACATGTCCCAACATGTTG
CTTGTACAGGGTTGTACAAC

Fig. 4

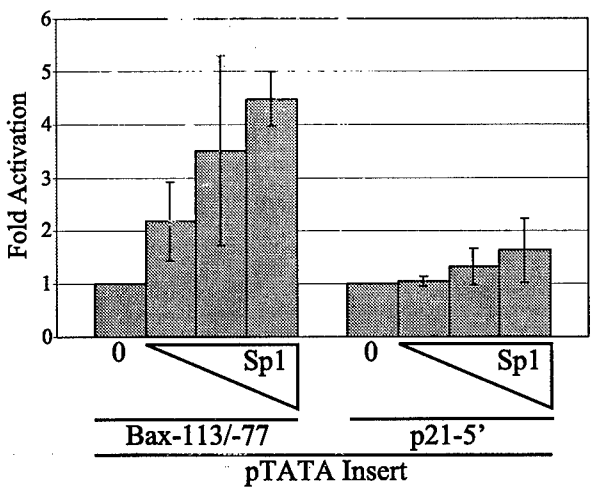

Fold Activation
6
5
4
3
2
1
0
0
Sp1
0
Sp1
Bax-113/-77
p21-5'
pTATA Insert

Fig. 5

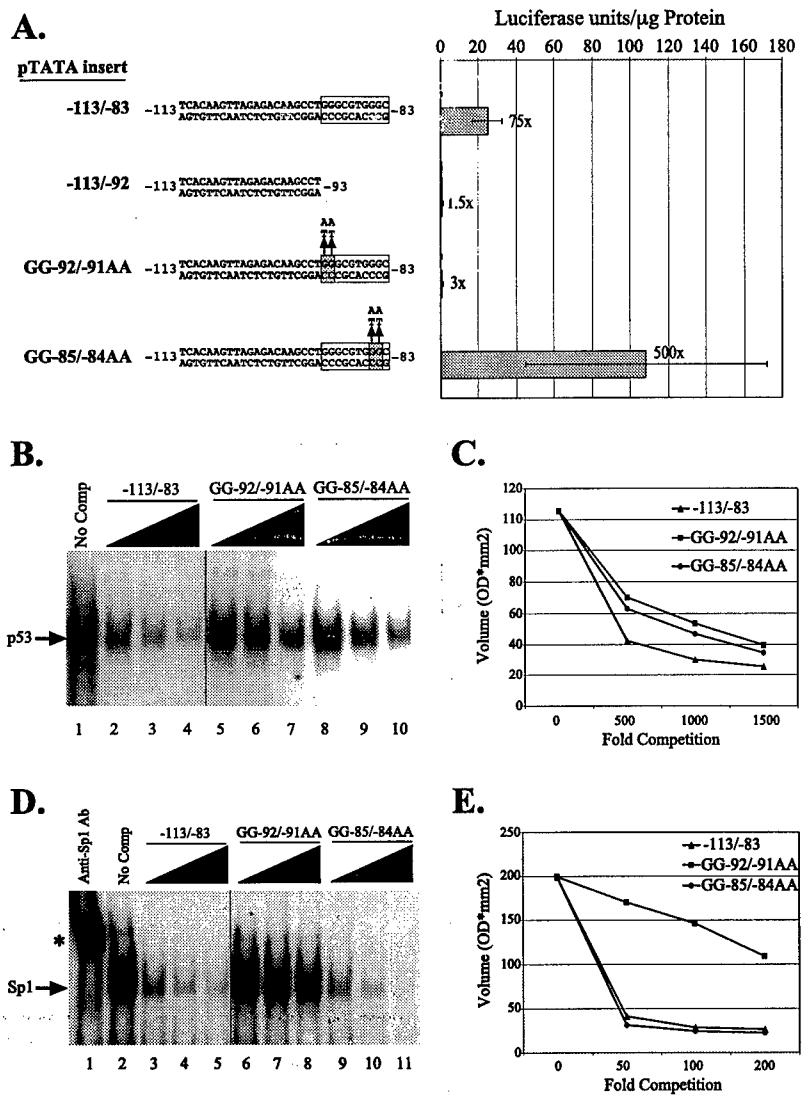

A.
pTATA insert
-113/-83
-113/-92
GG-92/-91AA
GG-85/-84AA
Luciferase units/μg Protein
0 20 40 60 80 100 120 140 160 180
75x
1.5x
3x
500x
B.
No Comp
-113/-83
GG-92/-91AA
GG-85/-84AA
p53
1 2 3 4 5 6 7 8 9 10
C.
-113/-83
GG-92/-91AA
GG-85/-84AA
Volume (OD*mm2)
Fold Competition
D.
Anti-Sp1 Ab
No Comp
-113/-83
GG-92/-91AA
GG-85/-84AA
Sp1
1 2 3 4 5 6 7 8 9 10 11
E.
-113/-83
GG-92/-91AA
GG-85/-84AA
Volume (OD*mm2)
Fold Competition

Fig. 6

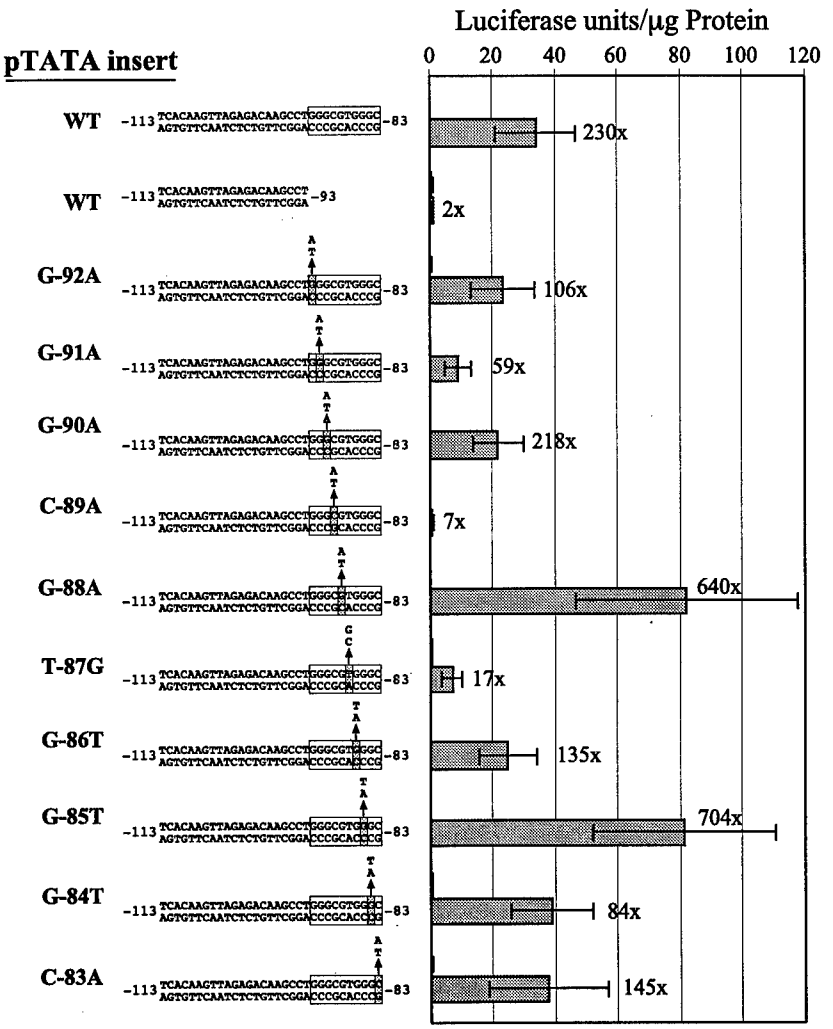

Luciferase units/µg Protein
0
20
40
60
80
100
120
pTATA insert
WT
-113 TCACAAGTTAGAGACAAGCCTGGGCGTGGGC -83
AGTGTTCAATCTCTGTTCGGACCCGCACCCG
230x
WT
-113 TCACAAGTTAGAGACAAGCCT -93
AGTGTTCAATCTCTGTTCGGA
2x
G-92A
106x
G-91A
59x
G-90A
218x
C-89A
7x
G-88A
640x
T-87G
17x
G-86T
135x
G-85T
704x
G-84T
84x
C-83A
145x

Fig. 7

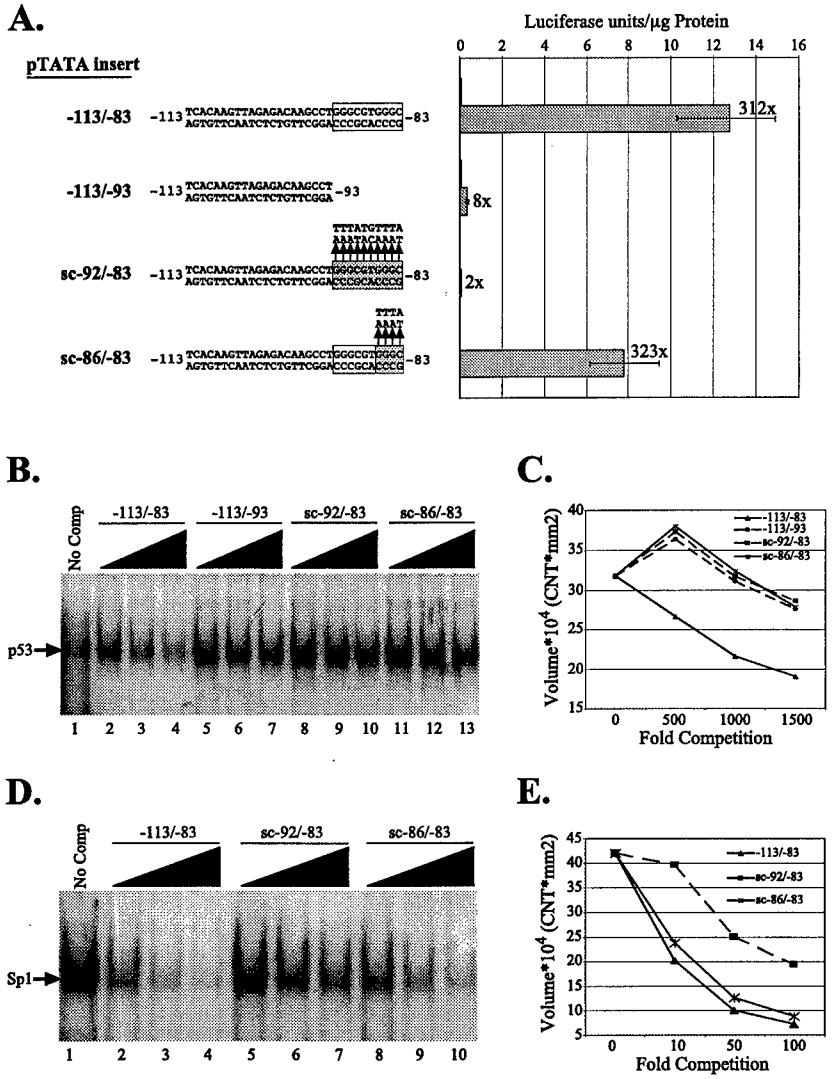

A.
pTATA insert
-113/-83
-113/-93
sc-92/-83
sc-86/-83
Luciferase units/µg Protein
312x
8x
2x
323x
B.
No Comp
-113/-83
-113/-93
sc-92/-83
sc-86/-83
p53
C.
Volume*10^4 (CNT*mm2)
Fold Competition
D.
Sp1
E.

Fig. 8

A high-affinity Sp1 binding site is located within the p53-response element of the human *bax* promoter.

Edward C. Thornborrow and James J. Manfredi. Derald H. Ruttenberg Cancer Center and Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY.

The tumor supressor protein p53 has been shown to mediate transcriptional activation of many of its target genes via a response element containing two consensus half-sites, each half-site consisting of PuPuPuC(A/T)(A/T)GPyPyPy. In contrast, activation by p53 of the element in the human *bax* promoter requires a cooperative interaction between three adjacent half-sites (Thornborrow and Manfredi, 1999, J. Biol. Chem. 274, 33747). The half-site most proximal to the start site of transcription contains the sequence 5'-GGGCGTGGGC-3', which closely resembles the DNA-binding consensus sequence for the transcription factor Sp1. Electrophoretic mobility shift assays (EMSAs) using an oligonucleotide containing the p53-response element derived from the *bax* promoter as radiolabeled probe demonstrated that a factor from nuclear extracts bound to this probe and this DNA-protein complex is supershifted by an anti-Sp1 antibody. Competition experiments using oligonucleotides corresponding to a number of well-characterized p53-response elements, including those from the *p21*, *cyclin G*, *IGF-BP3*, *mdm-2*, and *gadd45* genes, demonstrated that the binding of Sp1 to the p53 response element of the *bax* promoter was specific. Further, competition experiments with an oligonucleotide containing the Sp1 DNA-binding consensus sequence showed that the affinity of Sp1 for the p53 response element of *bax* is equal to if not greater than that of Sp1 for its consensus sequence. Deletion of the Sp1 binding site from the bax element results in a loss of demonstrable p53-dependent transcriptional activation in luciferase reporter assays. These results suggest that Sp1 and/or other Sp family members with similar DNA-binding characteristics, are important in regulating the p53-dependent transcriptional activation of the human *bax* gene.

Cdc25C is a target for sequence-specific repression by the tumor suppressor protein p53.

Selvon St. Clair, Abhilash Padi, Michelle R. Denburg, and James J. Manfredi. Derald H. Ruttenberg Cancer Center and Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY.

The dual specificity phosphatase Cdc25C mediates cell cycle progression into mitosis and has been implicated as a key player in the G2 checkpoint in response to DNA damage. A binding site for p53 in the human *cdc25C* promoter is sufficient to confer p53-dependent activation. However, induction of p53 down-regulates expression of endogenous *cdc25C* RNA and protein. The element responsible for this p53-dependent repression is shown to consist of the p53 binding site and an overlapping binding site for Sp1. A 2 bp mutation outside the p53 binding site inhibits both the binding of Sp1 *in vitro* and p53-dependent repression in cells. These results are consistent with p53 repressing *cdc25C* expression by blocking the binding to the promoter of another factor, most likely Sp1. Repression of *cdc25C* by p53 is proposed to be an additional mechanism for p53-dependent arrest in response to DNA damage. Further this is the first demonstration of p53-dependent down-regulation of a physiologically relevant target that requires sequence-specific DNA binding by p53.

CIS-ACTING PROMOTER ELEMENTS REGULATE GENE EXPRESSION MEDIATED BY THE TUMOR SUPPRESSOR p53

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Genetic alteration of p53 resulting in loss-of-function is a common event in many human cancers. In contrast, the majority of human breast carcinomas express a wild-type p53 protein. p53 is a transcription factor which exerts its effects by regulating the expression of particular target genes. Alterations in the expression of factors that regulate p53-dependent transcription may contribute to breast carcinogenesis in which the p53 itself is wild-type. As a first step in identifying such factors, three p53-dependent target genes have been identified in which additional sequence elements besides p53 binding sites are involved in the transcriptional response to p53.

Two response elements for p53 have been identified in the promoter encoding the cyclin-dependent kinase inhibitor p21. Deletion analysis demonstrated that promoter constructs containing only the downstream 3' site were robustly activated by p53 and yet a single copy of the 3' site placed upstream of a minimal promoter conferred only weak p53-dependent activation. A sequence element has been identified in the *p21* promoter which cooperates with the 3' site to activate transcription. These results argue that in the context of the *p21* promoter the 3' site must synergize with another activator elements to mediate p53-dependent activation. Studies are underway to identify the factor that interacts with this element.

The transcription factor Sp1 was shown to bind in a sequence-specific manner to the p53 response element in the human promoter for the gene that encodes the pro-apoptotic protein Bax but not to binding sites for p53 in other target genes. Deletion of the Sp1 binding site from the *bax* element resulted in a loss of demonstrable p53-dependent transcriptional activation in luciferase reporter assays suggesting that Sp1 and/or other Sp family members with similar DNA-binding characteristics, are important in regulating the p53-dependent transcriptional activation of the human *bax* gene.

The dual specificity phosphatase Cdc25C mediates cell cycle progression into mitosis. Induction of p53 down-regulates expression of endogenous *cdc25C* RNA and protein. The element in the *cdc25C* promoter that is responsible for this p53-dependent repression is shown to consist of a p53 binding site and an overlapping binding site for Sp1. A 2 bp mutation outside the p53 binding site inhibits both the binding of Sp1 *in vitro* and p53-dependent repression in cells. These results are consistent with p53 repressing *cdc25C* expression by blocking the binding to the promoter of another factor, most likely Sp1. Repression of *cdc25C* by p53 is proposed to be an additional mechanism for p53-dependent arrest in response to DNA damage. Further this is the first demonstration of p53-dependent down-regulation of a physiologically relevant target that requires sequence-specific DNA binding by p53.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7336 supported this work.

TRANSCRIPTIONAL REGULATION OF A p53 RESPONSE ELEMENT IN THE p21 PROMOTER IS DEPENDENT ON AN UPSTREAM CIS-ACTING ELEMENT.

L. Resnick-Silverman and J.J. Manfredi
Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York NY

The tumor suppressor protein p53 has been implicated in the cellular response to stress and mediates either growth arrest or apoptosis depending upon particular cellular conditions. Induction of expression of p53 results in transcriptional activation of particular genes, most notably that which encodes the cyclin-dependent kinase inhibitor p21. Upregulation of p21 has been shown to play a critical role in p53-mediated growth arrest. Two response elements for p53 have been identified in the promoter encoding the cyclin-dependent kinase inhibitor p21 an upstream or 5′ site and a downstream or 3′ site. Deletion analysis demonstrated that promoter constructs containing only this downstream 3′ site were robustly activated by p53 and yet a single copy of the 3′ site placed upstream of a minimal promoter conferred only weak p53-dependent activation. In contrast, a single copy of the 5′ site is capable of conferring robust p53-dependent activation. A sequence element has been identified in the *p21* promoter which cooperates with the 3′ site to activate transcription. These results argue that in the context of the *p21* promoter the 5′ site is sufficient for transcriptional activation by p53 but the 3′ site must synergize with another activator element to mediate p53-dependent activation.